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Confirmatory analysis of firocoxib in bovine milk by rapid resolution liquid chromatography tandem mass spectrometry

Geraldine Dowling[a,*], Pasquale Gallo[b], Liam Regan[a]

[a] The State Laboratory, Backweston Laboratory Complex, Young's Cross, Celbridge, Co. Kildare, Ireland
[b] Istituto Zooprofilatico Sperimentale del Mezzogiorno, Via Salute 2, 80055 Portici (Naples), Italy

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ABSTRACT

A rapid method has been developed to analyse for firocoxib (FIRO) residue in bovine milk. Milk samples were extracted with acetonitrile and sample extracts were purified on Evolute™ ABN solid phase extraction cartridges. Aliquots were analysed by rapid resolution liquid chromatography tandem mass spectrometry (RRLC–MS/MS). The method was validated in bovine milk, according to the criteria defined in Commission Decision 2002/657/EC. The decision limit (CCα) was 1.18 ng/mL and for the detection capability a (CCβ) value of 2.02 ng/mL was obtained. The measurement uncertainty of the method was 27%. Fortifying bovine milk samples ($n = 18$) in three separate assays, show the accuracy of the method to be between 96 and 105%. The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (5, 7.5 and 10 ng/mL) was less than 11% respectively.

1. Introduction

Firocoxib (3-cyclopropymethoxy-5,5-dimethyl-4-[4-(methyl sulfonyl) phenyl]-2-(5H)-furanone) is a non-steroidal anti-inflammatory drug (NSAID). The molecular structure of this compound is shown in Fig. 1. NSAIDs are used widely in veterinary medicine in the treatment of food producing animals. FIRO gives therapeutic efficacy due to inhibition of prostaglandin synthesis via selective binding to the type II cycloxygenase (COX-II) isoenzyme [1,2]. A survey involving 2000 veterinarians reported that 93% of veterinarians use NSAIDs in food producing animals and dairy practitioners reported the most frequent use [3]. Overall NSAIDs are an important group of compounds which are routinely used for the treatment of food producing animals [4]. A survey in 1995 reported that NSAIDs were the second most prescribed class of drugs after microbials for dairy practitioners [5]. In 2008 a study reported the increased incidence of residue violations for NSAIDs in cattle [6] in the past 10 years. According to EU law, all substances for veterinary use need to be included in Annexes I–III of Regulation 2377/90 [7]. This regulation establishes lists of compounds that have a fixed MRL (Annex I), that need no MRL (Annex II) or that have a provisional MRL (Annex III). FIRO is a compound that has been included in Annex I and has a maximum residue limit (MRL) established only in equine tissues. Substances that have no MRL established are prohibited for use in food producing animals. FIRO has no MRL established in bovine species. It is anticipated that due to the large increase in NSAID use in recent years that this substance may be used to treat food producing animals other than equines. Off label application of veterinary drug compounds to cows that produce milk for human consumption in the Republic of Ireland and the European Union is illegal. In Ireland in 2007, Ivermectin, a veterinary drug which is licensed in liver, kidney and fat of all mammalian food producing species but not authorised in animals that produce milk for human consumption was found in milk by the National Reference Laboratory for Avermectins in Ireland. The finding of this substance in milk is illegal within the EU. Firocoxib is a newly licensed NSAID in horses [8] and has become available on the market under the trade name of Previcox since 2007 [9] and Equioxx since 2008 [10]. Firocoxib cannot be used in mares in which milk is intended for human consumption. Firocoxib has been shown to be comparable in efficacy to meloxicam and carprofen [9] and also been shown to be comparable in efficacy to phenylbutazone [11]. In the case of carprofen and meloxicam these substances are licensed for use in horses and cattle, therefore it cannot be excluded that Firocoxib would not be used in cattle. As in the case of ivermectin, there is a need to anticipate the requirements of the future where risks could occur due to the administration of Firocoxib to milk producing species. Therefore the development of an analytical method at the National Reference Laboratory for NSAIDs in Ireland was undertaken to provide an analytical tool to monitor for this substance.

* Corresponding author.
E-mail address: Geraldine.Dowling@statelab.ie (G. Dowling).

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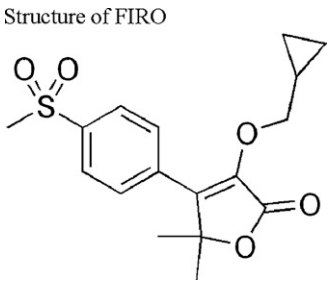

Fig. 1. Molecular structure of FIRO.

Longterm exposure to NSAIDs has caused kidney tumors in mice and liver tumors in rats [12]. It has been reported in recent years that the COX-II inhibitor class of NSAIDs of which FIRO is a member has been implicated in cardiovascular harm in humans [13,14]. Firocoxib shows the same undesirable side effects [9] as other NSAIDs (diarrhoea, mouth lesions and lethargy) therefore monitoring of its illegal use in milk producing animals is important for consumer protection.

There are very limited methods for the determination of FIRO in food producing animals and no methods for the determination of this substance in animal products.

Plasma of dogs and horses have been diluted with water and samples were purified using Waters™ HLB solid phase extraction cartridges and analysed by LC-UV [15]. Urine and plasma from dogs and horses was diluted with an aqueous solution of 5% acetic acid and passed through a Waters Oasis HLB™ 96-well solid phase extraction plate and analysed by liquid chromatography tandem mass spectrometry (LC–MS/MS) [16].

This method involves the addition of acetonitrile to bovine milk followed by clean-up using Evolute™ ABN solid phase extraction (SPE) cartridges and analysis by RRLC–MS/MS. To the best of our knowledge there are no methods published for the determination of FIRO in bovine milk. The objective of this study was to develop and validate a rugged, sensitive, selective and efficient method for the analysis of FIRO in bovine milk.

2. Experimental

2.1. Materials and reagents

Water, ethanol, ethyl acetate, methanol, acetonitrile, acetic acid, hydrochloric acid (37%), n-hexane and iso-octane (HiPerSolv grade) were obtained from BDH (Merck, UK). FIRO was given as a gift from Merial (Saint-Vulbas, France). Primary stock standard solution of FIRO (stable for 12 months was prepared in ethanol at a concentration of 1 mg/mL). Intermediate single standard solution of FIRO (stable for 6 months as standard injected throughout 6 months period gave similar results) was prepared in methanol at a concentration of 10 μg/mL. FIRO standard fortification solution (stable for 6 months as standard injected throughout 6 month period gave similar results) was prepared in methanol at a concentration of 500 ng/mL from the 10 μg/mL intermediate stock solution. All standards were stored at 4 °C in the dark. Isolute™ Evolute™ ABN 50 μm solid phase extraction cartridges (10 mL, 100 mg) were obtained from Biotage (Biotage, UK). Methanol:water (10:90, v/v) and 10 mM ascorbic acid were used as solid phase extraction wash solvents. n-Hexane:diethyl ether (50:50, v/v) was used as the solid phase extraction elution solvent. Injection solvent was water:acetonitrile (90:10, v/v).

Table 1
LC gradient profile for determination of FIRO.

Time (min)	Component A (%)	Component B (%)
0.0	90	10
0.4	90	10
1.0	85	15
3.1	20	80
4.1	20	80
4.7	90	10
6.5	90	10

Component A: water containing 0.001 M acetic acid + acetonitrile (90 + 10, v/v) and Component B: acetonitrile.

2.2. LC–MS/MS conditions

The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B Binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven (Agilent Ireland). FIRO was chromatographed on a 1.8 μm Agilent Eclipse Plus C_{18} column (2.1 mm × 50 mm) (Agilent, Ireland) and the column temperature was maintained at 55 °C. A gradient was applied with water and acetonitrile (90:10, v/v + 0.001 M acetic acid) (A) and acetonitrile (B). The flow rate throughout the chromatographic analysis was 0.75 mL/min and the following gradient was applied: 0 min, 90% A; 0.4 min, 90% A; 1.0 min, 85% A; 3.1 min, 80% B; and 4.7 min, 90% A. The column was regenerated for 1.8 min before injection (Table 1). The total run time was 6.5 min. The injection volume was 15 μL. The mass spectrometer used was a QTRAP 4000 with a TurboIonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.4.2 of Analyst software. The described LC–MS/MS system was shown to be suitable for the analysis of FIRO (Figs. 2–3).

2.3. MS/MS parameters

The analysis was performed using positive ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. Two transitions were used and the collision voltages were optimised as shown (Table 2). Each transition was performed with a 13 ms dwell time and a pause time of 3 ms. The MS/MS detector conditions were as follows: Ion mode electrospray positive; curtain gas 45 psi; ion spray voltage 4400 V; temperature 650 °C; ion source gas 1 70 psi; ions source gas 2 70 psi; Interface heater on; entrance potential 10 V; Resolution Q1 unit; Resolution Q3 unit; CAD gas = high.

2.4. Milk samples

Untreated milk from 8 individual cows was obtained by veterinary inspectors and milk (5 different brands of whole milk) obtained from a local supermarket were used as negative controls. The milk was analysed separately and no detectable residues of FIRO were found. Milk samples previously analysed were pooled together and separated into 50 mL aliquots and stored at −20 °C and used as negative controls in the experiments.

2.5. Sample extraction and clean-up

Milk samples (5 mL) were aliquoted into 50 mL polypropylene tubes. Samples were fortified at levels corresponding to 5, 7.5 and 10 ng/mL by adding 50, 75 and 100 μL portions of a 500 ng/mL solution of FIRO. After fortification, samples were held for 15 min prior to extraction. Acetonitrile (5 mL) was added and the samples were vortexed (30 s), centrifuged (3500 rpm, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube.

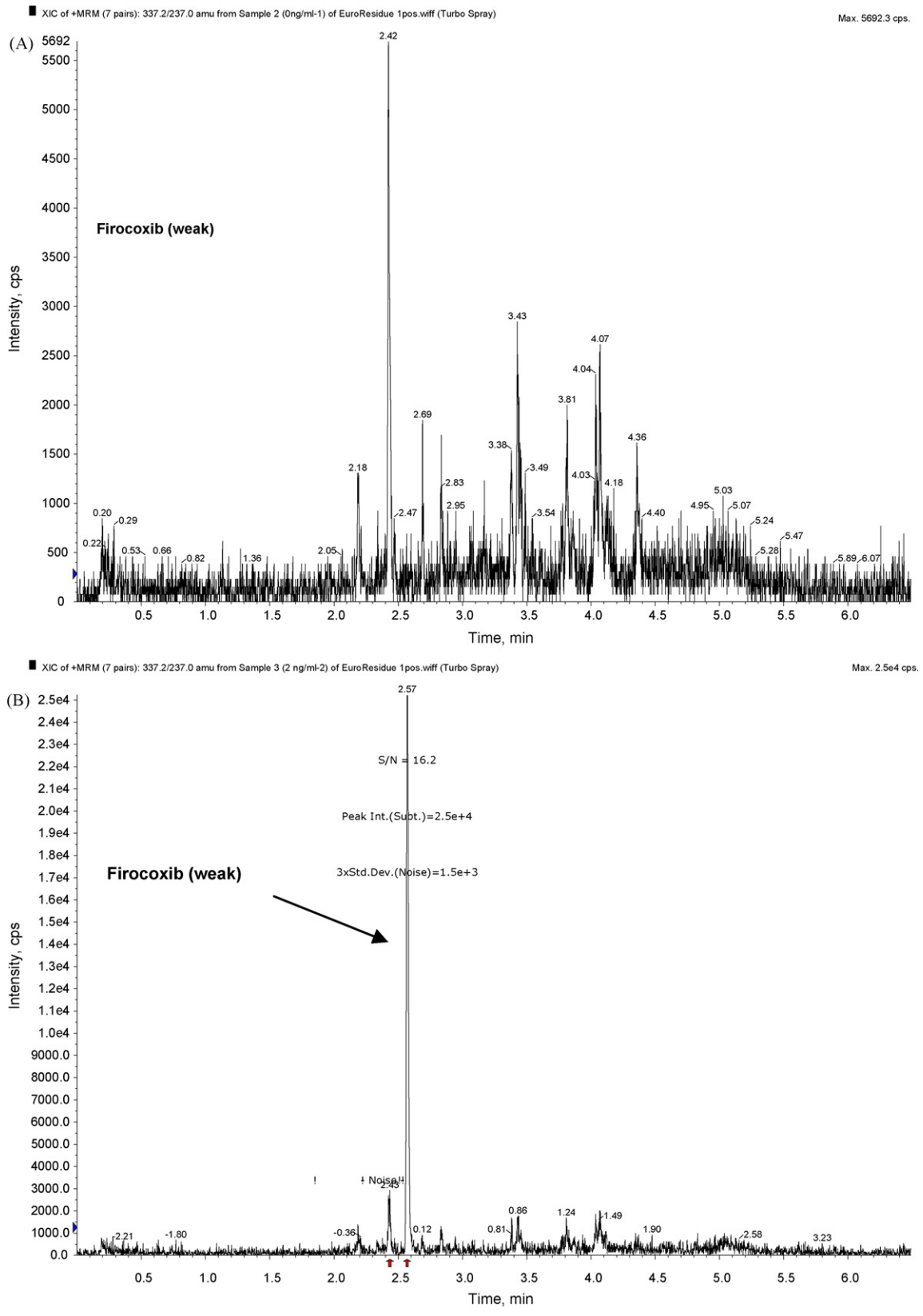

Fig. 2. Chromatogram of negative control bovine milk (A) and negative control bovine milk fortified with 2 ng/mL of FIRO (B).

The sample pellet is re-extracted with 5 mL of acetonitrile and the supernatants are combined. 10 mM ascorbic acid (20 mL) and 1 M hydrochloric acid (0.2 mL) were added to the extracts and the pH of the samples were checked to ensure they were at pH 3 before proceeding to the solid phase extraction stage. The sample extracts were purified by SPE using Evolute™ ABN™ SPE cartridges. Sample extracts were loaded onto the cartridges (preconditioned with methanol (3 mL) and ascorbic acid (3 mL). The samples were loaded onto cartridges under gravity. The cartridges were washed with methanol:water (10:90, v/v) (2 mL). The car-

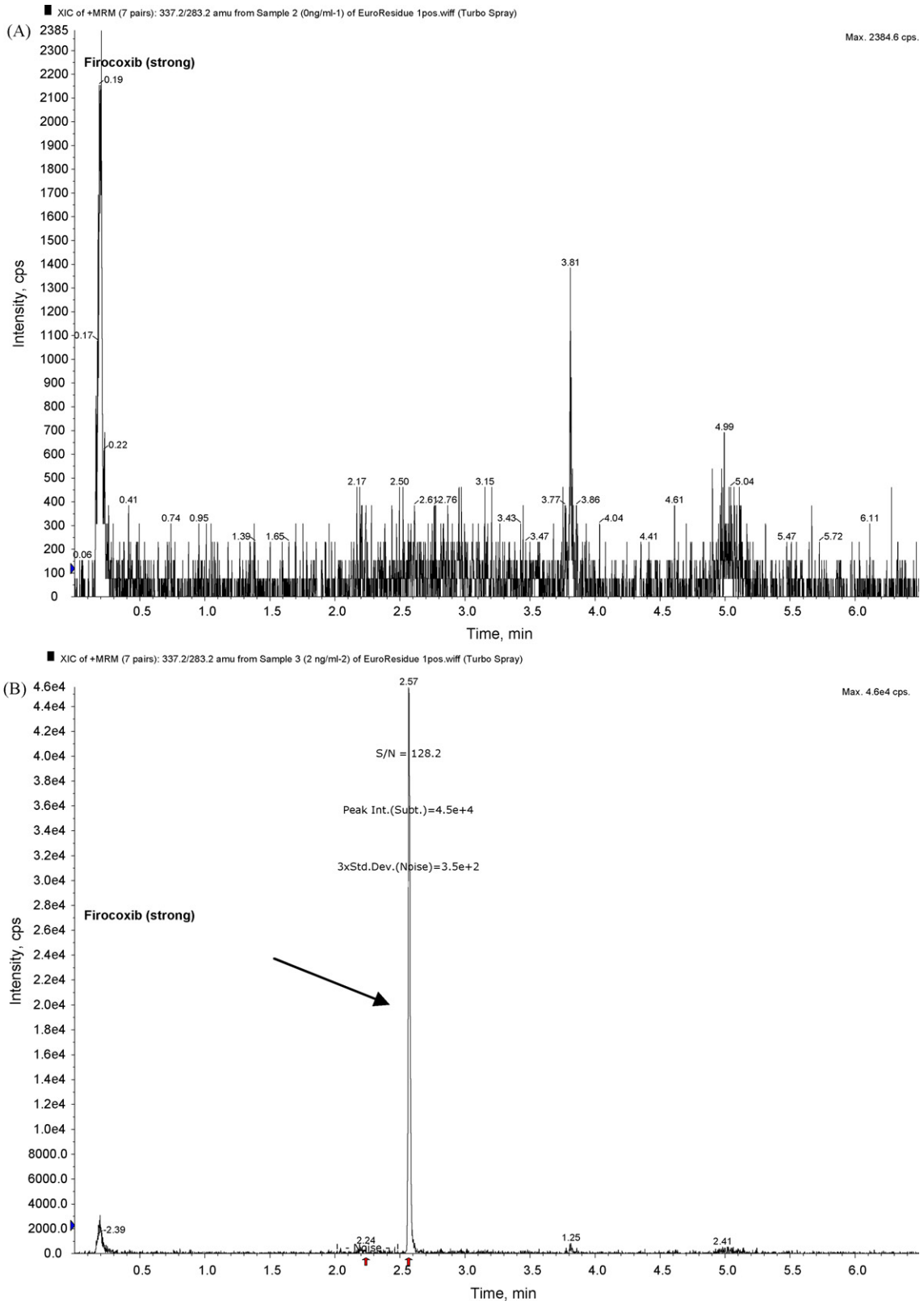

Fig. 3. Chromatogram of negative control bovine milk (A) and negative control bovine milk fortified with 2 ng/mL of FIRO (B).

tridges were dried under vacuum (15 min). The cartridges were eluted with n-hexane:diethyl ether (50:50, v/v) (2 × 2 mL). The eluates were reduced to dryness under nitrogen without heat before re-dissolving in 150 μL water:acetonitrile (90:10, v/v) and vortexed (1 min). An aliquot (15 μL) is injected on the LC column.

2.6. Matrix-matched calibration

Matrix-matched calibration curves were prepared and used for quantification. Control milk previously tested and shown to contain no residues was prepared as Section 2.4. One control milk sample was used for each calibration standard level. Milk samples (5 mL)

Table 2
MS/MS parameters for the determination of FIRO.

Compound	Transition	Declustering potential [V]	Collision [V]	CXP [V]
FIRO	337.2 > 283.2 (strong)	71.24	13	19
	337.2 > 237.0 (weak)	71.24	23	16

were aliquoted into 50 mL polypropylene tubes. Samples were fortified at levels corresponding to 0, 2, 5, 7.5, 10 and 20 ng/mL by adding 0, 20, 50, 75, 100 and 200 μL portions of a 500 ng/mL standard solution of FIRO. After fortification, samples were held for 15 min prior to the extraction procedure as described in Section 2.5. Calibration curves were prepared by plotting the peak area as a function of analyte concentration (0–20 ng/mL) to quantify samples.

2.7. Method validation

For estimation of accuracy, blank milk samples were fortified with FIRO at 5, 7.5 and 10 ng/mL. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions. For the estimation of the precision of the method, repeatability and within-laboratory reproducibility was calculated. The decision limit (CCα) of the method was calculated according to the calibration curve procedure using the intercept (value of the signal, *y*, where the concentration, *x* is equal to zero) and 2.33 times the standard error of the intercept for a set of data with 6 replicates at 3 levels. The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα.

3. Results and discussion

3.1. Preliminary experiments

The LC–MS/MS method was developed to provide confirmatory data for the analysis of bovine milk for FIRO. The MS/MS fragmentation conditions were investigated and collision energies were optimised. For a method to be deemed confirmatory four identification points are required. These identification points can be obtained by monitoring one precursor ion (parent mass) and two daughters (corresponding to strong and weak ion).

FIRO was chromatographed on a 1.8 μm Agilent Eclipse Plus C_{18} column with retention time of 2.57 min.

3.2. Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [17] covering specificity, calibration curve linearity, recovery (accuracy), precision, decision limit (CCα) and detection capability (CCβ).

3.2.1. Specificity

The technique of LC–MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, a variety of milk samples were fortified with the FIRO and non-fortified samples were also analysed. No interfering peaks were observed at the retention time for FIRO (Fig. 2). Additionally samples were fortified with 2.0 ng/mL of other NSAIDs which included flunixin (FLU), carprofen (CPF), meloxicam (MLX), oxyphenylbutazone (OXYPHEN) and diclofenac (DCF). No interferences were observed in the retention window of FIRO in chromatograms when fortified with these substances.

3.2.2. Linearity of the response

The linearity of the chromatographic response was tested with matrix-matched curves using 6 calibration points in the concentration range of 0–20 ng/mL. The regression coefficients (r^2) for all the calibration curves used in this study were ≥0.99.

3.2.3. Accuracy

The accuracy of the method was determined using bovine milk samples fortified at 5.0, 7.5 and 10.0 ng/mL. Mean corrected recovery ($n = 18$) of the analyte, determined in three separate assays (Table 3) was between 96 and 105%.

3.2.4. Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (5, 7.5 and 10 ng/mL) was less than 11% (Table 3).

3.2.5. CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1% is applied. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of 1-β, were β = 5%. CCα and CCβ were calculated using the intercept (value of the signal, *y*, were the concentration, *x* is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (5.0, 7.5 and 10.0 ng/mL). Blank milk was fortified at 1, 1.5 and 2 times the minimum required performance level of 5 ng/mL set for FIRO. CCα is the concentration corresponding to the intercept +2.33 times the standard error of the intercept. CCα value of 1.18 ng/mL was achieved for FIRO. CCβ is the concentration corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e. the intercept +3.97 times that standard error of the intercept). CCβ value of 2.02 ng/mL was achieved for FIRO.

3.2.6. Measurement uncertainty

According to SANCO/2004/2726 rev 1 the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [18]. For the calculation of the extended uncertainty a safety factor is required. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the CCα, corresponding to a confidence level of 99%. As the only source of variation during the validation was the different days and different milk sourced from different animals it was decided to use a safety factor of 3.0 instead of

Table 3
Intra- and inter-assay variation for recovery of FIRO from milk.

Analyte	Fortification level (ng mL^{-1})	Recovery (%)	Within Run CV (%)	Between Run CV (%)	Total CV (%)
FIRO	5	104.4	2.9	2.5	3.9
	7.5	96.3	8.8	7.1	11.3
	10	105.2	8.9	5.2	10.4
Combined variance	5, 7.5, 10				9.1

2.33. The measurement uncertainty of the method was estimated at 27%. This was determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.

4. Conclusions

A relatively fast, simple, sensitive and selective RRLC–MS/MS method for the detection of FIRO in bovine milk has been developed. There is no published confirmatory method for the determination of FIRO in bovine milk that is validated according to Commission Decision 2002/657/EC [17]. This is the first time that milk extracts have been purified using Evolute™ ABN solid phase extraction cartridges for the determination of FIRO and the first time that FIRO has been analysed using RRLC–MS/MS. The method performs very well in terms of accuracy and within-laboratory reproducibility. In monitoring for this substance at our laboratory in 2008 it was possible to detect the precursor ion and two daughter ions (at 2 ng/mL) in multiple reaction monitoring mode in the lowest standard in the matrix-matched curve. Furthermore the product ion ratio requirement was also met. The method meets the requirements for a confirmatory method according to 2002/657/EC. The method has been carried out by different analysts under varying environmental conditions and the method was shown to be robust.

The objective of the work to develop and validate a method for this residue in bovine milk in Ireland at low ng/mL levels and validate according to the requirements in Commission Decision 2002/657/EC therefore has been achieved successfully.

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